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PRINCIPAL INVESTIGATOR: Dr. Michael Hilinski

CONTRACTING ORGANIZATION: The University of Virginia
Charlottesville, VA 22903-4833

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E-Mail: mh6cu@virginia.edu

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14. ABSTRACT

RSK (p90 Ribosomal S6 kinase) is critical for breast cancer proliferation and thus a promising target for therapeutic intervention. A highly specific inhibitor of RSK, called SL0101, was previously discovered but found to possess poor biological stability and potency. The purpose of this project is to identify a drug for breast cancer based on SL0101 that works by inhibiting RSK, by designing and chemically synthesizing analogues of SL0101 that improve on its biological stability and potency and ultimately evaluating them in a living human breast tissue model for anticancer activity. The major finding in this year of funding that are approximately ten times more potent than SL0101 at inhibiting the growth of breast cancer cell lines. This discovery represents a significant step toward the goal of identifying an analogue of SL0101 that could be used as a drug for breast cancer.

15. SUBJECT TERMS
breast cancer, p90 Ribosomal S6 kinase, RSK, kinase inhibitor, medicinal chemistry, cell permeability

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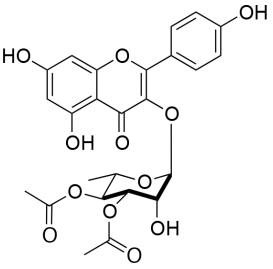
Introduction

The p90 ribosomal S6 kinase (RSK) family of serine/threonine protein kinases, comprising at least four isoforms (RSKs 1-4), has been shown by our laboratory to be critical for breast cancer cell proliferation (*1*–*3*). In 2005 our laboratory reported the first specific inhibitor of RSK, SL0101 (**1**, Figure 1) (*3*). SL0101 inhibits RSK in both the breast cancer cell line MCF7 and the normal breast cell line MCF-10A, but only inhibits the proliferation of the breast cancer cell line (*1*–*3*). This indicates that breast cancer cells have become dependent on RSK and thus identifies RSK as a potential new target for cancer therapeutics. SL0101, given its exquisite specificity for RSK, is an attractive lead compound for medicinal chemistry efforts aimed at discovering a breast cancer drug that acts by inhibiting RSK. However, SL0101 itself is not suitable for further development as a drug for two reasons. First, we have determined that SL0101 has a very short biological half-life in mice (0.4 h at 2.5 mg/kg IP). In order to develop a drug this half-life must be improved so that the drug persists in the patient long enough to act on RSK. Second, SL0101 is a potent RSK inhibitor (IC_{50} ~ 0.5 μM), but a much less potent inhibitor of the proliferation of MCF7 breast cancer cells (EC_{50} = 50 μM), suggesting that it does not readily pass through the cell membrane (*3*). In order to develop a drug, the potency against MCF7 cells must be improved. The scope of this project is to design and chemically synthesize analogues of SL0101, with the assistance of a computational model of SL0101 bound to RSK, that improve on these deficiencies and to evaluate them both in vitro and in vivo with the goal of identifying a new breast cancer drug that acts by inhibiting RSK. In addition, the best analogues will be evaluated in our new living human breast tissue model (*4*) to gain insights in to the role of RSK in breast cancer that might not be gleaned from in vitro and cell-based assays.



SL0101 (**1**)

Fig 1. SL0101, a highly specific inhibitor of p90 ribosomal S6 kinase (RSK)

Body

In the previous year of the project (Year 1) the in vitro biological stability of SL0101 analogues was improved by modifying the substitution on sugar portion of SL0101 to replace the biologically labile acetates at the 3' and 4' positions of the carbohydrate ring with relatively biologically inert carbamates (*5*). Importantly, the carbamate analogues retained specificity for RSK as measured by their selective inhibition of the growth of cancer over normal cell lines. As expected, this type of structural modification did not improve substantially the potency of the compounds for inhibition of RSK kinase activity (i.e. in vitro potency) nor for inhibition of cancer cell proliferation (i.e. ex vivo potency). The focus for Year 2 of the project therefore turned to improving the in vitro and ex vivo potency of analogues. This goal was aided by the crystal structure of SL0101 bound to the N-terminal kinase domain of RSK2 obtained in Year 1 (*6*). What follows is a detailed description of the results obtained relevant to the Statement of Work for Year 2.

Task 4: Design, synthesis, and biological evaluation of potentially more potent SL0101 analogues that append lipophilic groups from the flavone ring system, or related analogues. I will first prioritize analogues based on a computational model. I will then synthesize one or more analogues and evaluate their biological activity. If warranted, I will then synthesize additional related analogues. (Timeframe: months 9-24)

This task was completed within the allotted timeframe. Details for each subtask follow and experimental details are attached in the appendix. As reported previously, during Year 1 potential analogues, including the stated analogues "that append lipophilic groups from the flavone ring system" were docked into the SL0101 binding site on the NTKD of RSK2. However, the new structural information obtained from the crystal suggested that these analogues would in fact be worse inhibitors than SL0101, and therefore instead I chose to pursue the "related analogues" mentioned in the task.

4b. Synthesis of an initial analog or set of analogs prioritized based on modeling results. (months 12-15)

This task was completed within the allotted timeframe. The modeling results, a subset of which are shown in figure 2, suggested that structural changes to the A-ring of SL0101 could lead to analogs with improved binding to the NTKD of RSK2 and by extension improved ability to inhibit RSK activity. The

compounds were docked into the SL0101 binding site of the RSK2 NTKD using the Molecular Operating Environment (MOE) software package published by Chemical Computing Group (CCG) of Montreal, Canada. A docking score (a measure of the favorability of binding interactions) and RMSD (similarity of the binding pose to the binding pose of SL0101 as seen in the crystal structure) were generated for each compound. In this case, a lower docking score and RMSD indicate favorable binding in a similar orientation to SL0101. Therefore analogue **2**, having the lowest docking score of this subset of analogue candidates, was chosen as the initial synthetic target. Due to the commercial availability of styrene **6** a cross-metathesis strategy starting from known SL0101 intermediate **5** was chosen as an initial approach to A-ring analogues (Scheme 1). Several different conditions were attempted for the cross metathesis reaction (varying solvent and temperature) however in no case was the desired product **7** observed. Cross metathesis attempts with other alkenes were also unsuccessful (not shown).

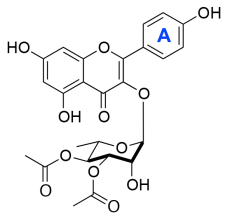
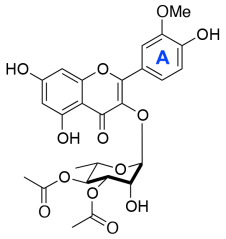
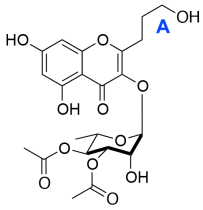
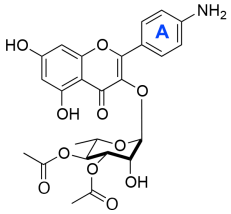
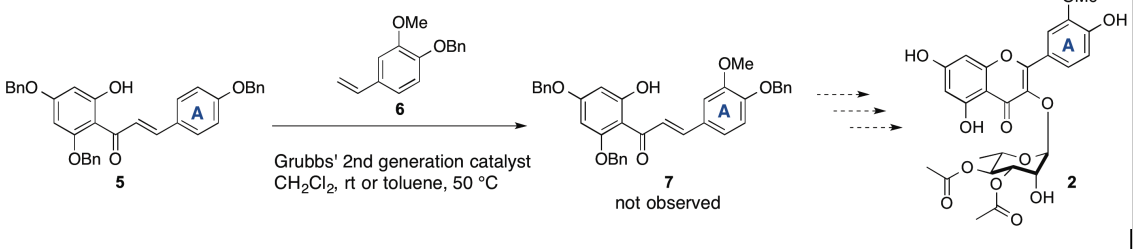
Compound #	Structure	Docking Score	RMSD
1		-7.96	1.63
2		-8.07	1.95
3		-7.74	3.32
4		-7.95	1.44

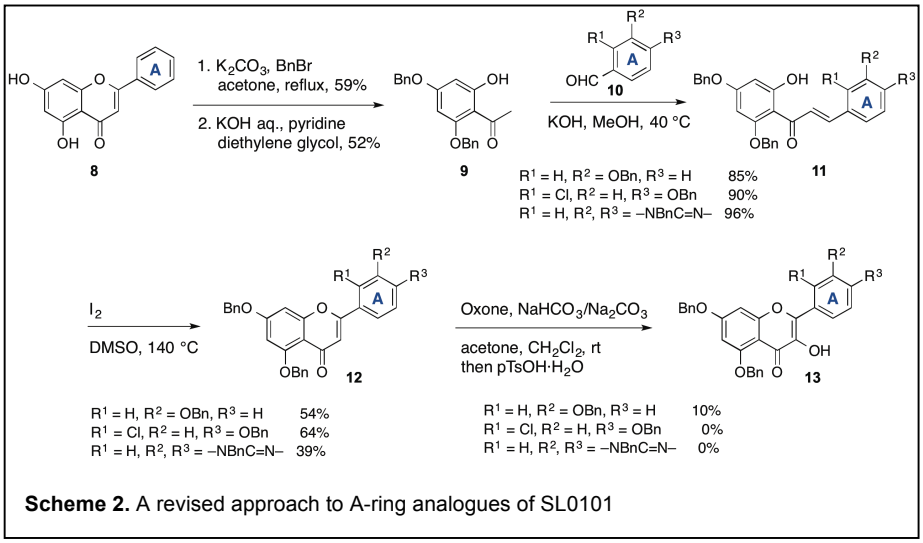
Figure 1. Docking results obtained for proposed analogues using the MOE software package.

In order to complete an initial A-ring analogue a revised synthetic strategy was employed (Scheme 2). The key step to install a new A-ring moiety was an aldol condensation between ketone **9** and aldehydes **10**. Initial aldehydes used in this reaction were selected based on commercial availability and the docking results for the proposed A-ring analogue (not shown). The initial aldol condensation proceeded smoothly and in high yield for all cases, providing enones **11**, which were then cyclized to provide flavones **12**. Unexpectedly hydroxylation to provide the completed "top piece" intermediates **13** gave none of the desired product for two of the modified flavones and only 10% yield for the third.

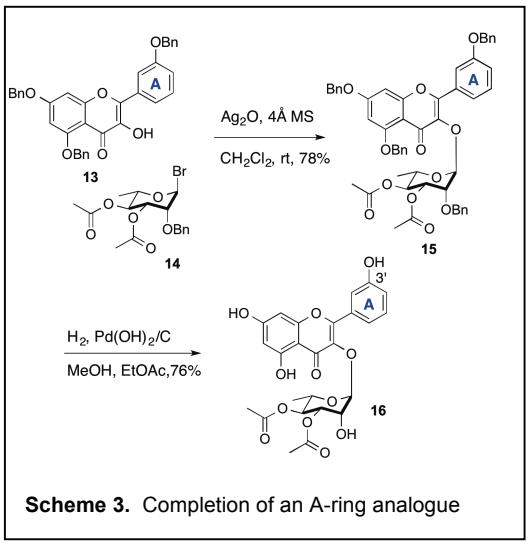


Scheme 1. An initial attempt at synthesizing A-ring analogues of SL0101 utilizing an olefin cross-metathesis strategy

Despite the poor yield for the previous step I was able to complete the synthesis of an initial A-ring analogue of SL0101 in which the phenolic hydroxyl group of the A-ring is relocated to the 3' position of the A-ring. Glycosylation of intermediate **13** followed by removal of the four benzyl protecting groups provided completed analogue **16** in good yields for both steps.



Scheme 2. A revised approach to A-ring analogues of SL0101



Scheme 3. Completion of an A-ring analogue

4c. In vitro evaluation of analog or analogs. (months 15-18)

I completed this task within the allotted timeframe. I evaluated A-ring analogue **16** in an in vitro kinase assay. It inhibited RSK2 kinase activity with an IC_{50} of 43.5 μM. Analogue **16** was 25-fold less potent than SL0101 (IC_{50} = 1.7 μM) in the same assay. This large decrease in potency was surprising since the docking results based on the crystal structure of SL0101 bound to RSK2 suggested that it should be roughly equipotent, if the ability of an analogue to bind to RSK2 correlates with inhibitory activity. This result suggests a need to reevaluate the use of docking scores for selection of SL0101 analogue targets.

4d. Evaluation of growth inhibition activity of analogs in human cancer MCF-7 and normal human MCF-10A cell lines. (months 15-18)

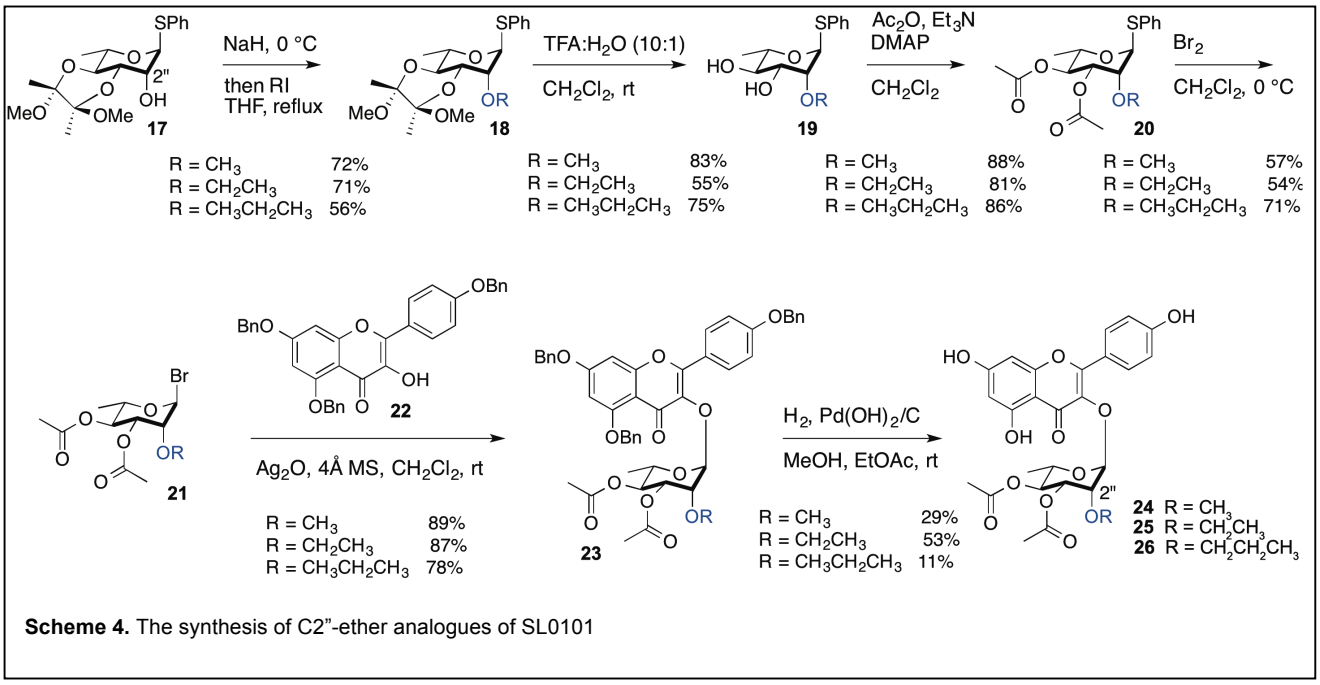
A preliminary evaluation of the ability of analogue **16** to inhibit the growth of MCF-7 cells indicated that it was substantially less potent than SL0101, showing essentially no effect on growth up to a concentration of 100 μM. As a consequence, I decided not to proceed with the further biological evaluation and instead focus on developing other analogues with the chance to be superior to SL0101 (see subtask 4e).

4e. If warranted, synthesis and biological evaluation of additional related analogs. (months 18-24)

I completed this task within the allotted timeframe. Due to the poor biological activity of analogue **16**, I decided to refocus my efforts on a series of analogues I first explored during Year 1 of the project. I previously demonstrated that the 2” hydroxyl group of the rhamnose portion of SL0101 could be replaced with a methyl ether (analogue **24**, Scheme 4) and that the new analogue is indistinguishable from SL0101 in its ability to inhibit RSK. The rationale behind the design of this analogue was that the crystal structure of SL0101 bound to the NTKD of RSK2 shows that the C2” hydroxyl group of SL0101 accepts a hydrogen bond from a nearby lysine, rather than using its hydrogen to donate a hydrogen bond. My hypothesis was that since the hydrogen of the hydroxyl group was not necessary, alkylation to form an ether would not be detrimental to an analogue’s ability to inhibit RSK. Furthermore, an ether at that position would increase the lipophilicity of the analogue overall and potentially increase its ability to enter cells. The outcome of this would be improved potency of the analogue in cell-based assays. Indeed, I disclosed in my last annual report that analogue **24** in initial proliferation assays with MCF-7 cells was roughly twice as potent as SL0101.

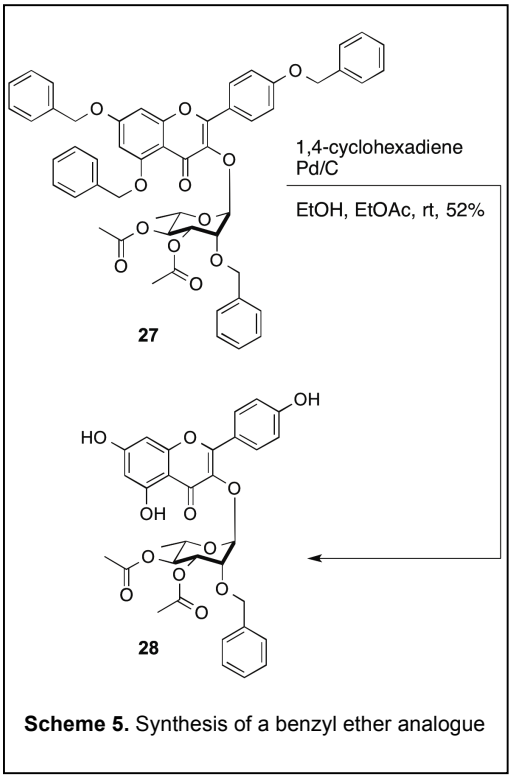
Based on these initial results I decided that additional analogues in this series were warranted to continue to try to improve the potency of SL0101 in cell-based assays. Toward this end I re-synthesized analogue **24** and synthesized two new analogues **25** and **26** (Scheme 4), incorporating ethyl and n-propyl ethers, respectively. The synthetic route used to access these analogues is shown in Scheme 4. The key diversification step, namely an alkylation of intermediate **17** with various alkyl halides to provide ethers **18**,

proceeded smoothly and in moderate to good yields. The remainder of the synthesis was unchanged from the synthetic route to SL0101, and proceeded without incident to provide completed 2" ether analogues **24**–**26**.



Scheme 4. The synthesis of C2"-ether analogues of SL0101

To add to the collection of C2" ether analogues of SL0101 I synthesized one additional analogue using a different synthetic route. Under transfer hydrogenation conditions I was able to selectively remove the phenolic benzyl protecting groups of known intermediate **27** while leaving the C2" benzyl ether intact, providing C2" ether analogue **28** (Scheme 5).



Scheme 5. Synthesis of a benzyl ether analogue

I tested all new analogues along with SL0101 for their ability to inhibit RSK activity in an in vitro kinase assay (Table 1). The methyl ether analogue **24** was as potent as SL0101 and analogs **25**, **26**, and **28** were moderately less potent. A trend was observed wherein potency decreased as the lipophilicity of the C2" ether substituent increased. I also tested the ability of all new analogs to inhibit the growth of the MCF7 breast cancer cell line. Gratifyingly, and consistent with the hypothesis that the ether analogues would have improved cell permeability, all of the new analogs were more potent than SL0101 at inhibiting the growth of the cancer cell line. A subset of the proliferation data for the most potent analogues is shown in Figure 2a. Analogue **26** inhibited the proliferation of MCF-7 cells with an IC_{50} of 8.7 μM, while the IC_{50} of analogue **28** was 6.9 μM. Given that the IC_{50} of SL0101 in this assay is typically between 50–100 μM, this means that the new analogues are approximately *10-fold more potent* than SL0101. This is highly significant in that the potency of both **26** and **28** is in the range needed to proceed with animal studies, achieving an important interim goal of this project. Analogues **26** and **28** were also evaluated for their ability to inhibit the growth of the normal breast cell line MCF-10A (Figure 2b). At the highest concentration tested, 25 μM, neither new analogue exhibited 50% inhibition. This is significant because an important

Compound	RSK2 IC_{50} (μM)
1	1.77 (1.45 to 2.18)
24	2.28 (1.58 to 3.27)
25	6.93 (5.09 to 9.43)*
26	10.7 (7.95 to 14.5)*
28	36.9 (27.0 to 50.2)*

Table 1. Potency of analogues in the in vitro kinase assay. IC_{50} is concentration needed for 50% inhibition; the 95% CI is shown in parentheses; n=2 in triplicate; * p <0.05

measure of the selectivity of an analogue for RSK over other kinases is its ability to selectively inhibit the growth of cancer cell lines over normal cell lines. In short, normal cell lines are not dependent on RSK activity for growth and therefore a selective RSK inhibitor should show a decreased ability to inhibit the growth of the normal cell line. These results suggest that from the standpoint of potency and selectivity for RSK, **26** and **28** are good candidates for in vivo evaluation.

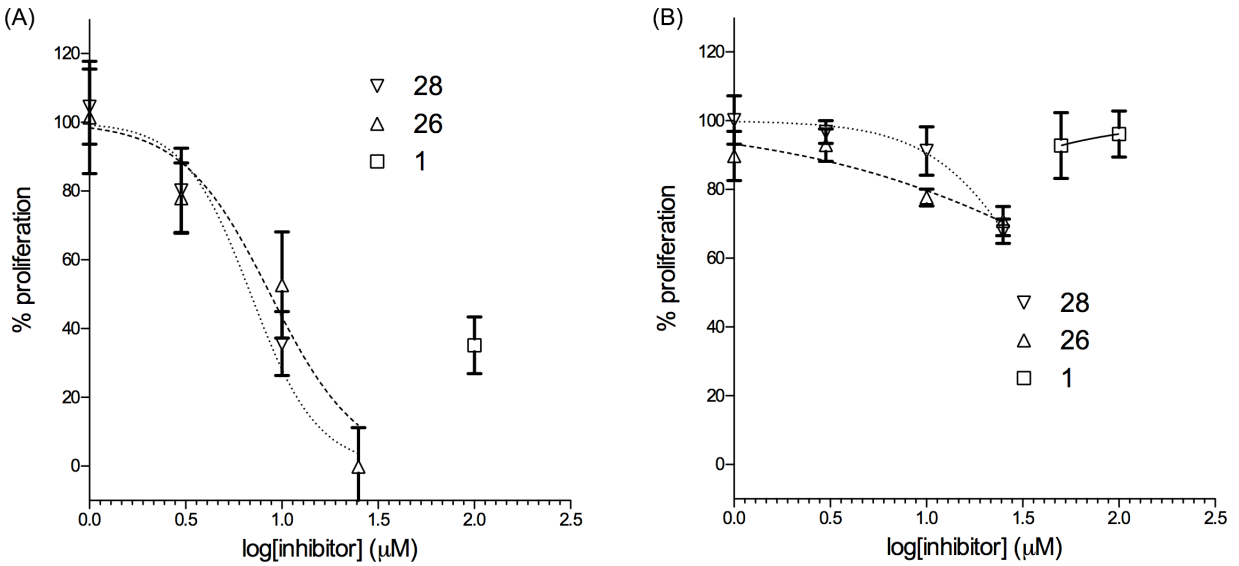


Figure 2. SL0101 and analogues **26** and **28** selectively inhibit MCF-7 over MCF-10A cell proliferation. (A) MCF-7 cells were treated with vehicle or the indicated concentration of SL0101, **26** and **28**. % Proliferation shown is normalized to treatment with vehicle. (B) MCF-10A cells were treated with vehicle or the indicated concentration of SL0101, **26** and **28**. % Proliferation shown is normalized to treatment with vehicle.

Task 5: In collaboration with Michelle Rudek-Renaut, evaluate the biological half-life of 1-3 analogs in CB17 SCID mice for both subcutaneous and intraperitoneal routes. (Timeframe: months 1-6 and 21-27)

5b. Evaluation of the biological half-life of 1-3 analogs in CB17 SCID mice for both subcutaneous and intraperitoneal routes. This will require 30 mice per analog to be able to collect 15, 30 min, 1, 3 and 6 h time points. (months 21-27)

This task is currently in progress. Analogues **26** and **28** and versions that incorporate the carbamate substitution previously shown to confer greater biological stability (not shown) are currently being synthesized in amounts (100-200 mg) that will support the indicated studies.

Training Plan:

Task 3: Regularly attend cancer and chemistry seminars. (Timeframe: 1-36 months)

I regularly attended seminars run by the University of Virginia Cancer Center and in the University of Virginia Chemistry Department during Year 2 of the project.

Task 5: Attend international meeting held in the US to present and discuss work. (Timeframe: 13-24 months)

Since I attended three meetings during Year 1 of the project rather than the planned one, I chose not to attend a meeting during Year 2.

Key Research Accomplishments

- The discovery of analogues of the RSK inhibitor SL0101 that are roughly ten times more potent than SL0101 at inhibiting the growth of breast cancer cells and are selective inhibitors of the growth of cancer vs. normal cell lines. These analogues are candidates for in vivo evaluation.

Reportable Outcomes

- Employment received based on training supported by this award: The P.I., Michael Hilinski, has been hired as an Assistant Professor in the Department of Chemistry at the University of Virginia starting in the Fall of 2013.

Conclusion

SL0101 is a promising lead compound for medicinal chemistry efforts to develop a breast cancer drug that works by targeting RSK. However it suffers from poor biological stability and potency, making it unsuitable for use as a drug. The discovery of analogues of SL0101 that are more biologically stable and that are more potent in cell-based assays as described in this report is thus highly significant as they overcome these deficiencies and therefore could find use as breast cancer drugs. In particular analogues **26** and **28** exhibit 10-fold better potency in cell-based assays than SL0101, which is sufficient potency to support their further evaluation in animal models of breast cancer. Overall, the work accomplished in this year of funding moves the science of RSK inhibitors closer to the goal of a breast cancer drug that works by inhibiting RSK.

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Appendices

none